Immunity, Vol. 9, 721–731, November, 1998, Copyright ©1998 by Cell Press

A Critical Role for Complement in Maintenance of Self-Tolerance

Andrey P. Prodeus,*§ Siegfried Goerg,*§
Li-Ming Shen,* Olga O. Pozdnyakova,*
Lisa Chu,* Elisabeth M. Alicot,*
Christopher C. Goodnow,† and Michael C. Carroll*‡
*The Center for Blood Research
Department of Pathology
Harvard Medical School
Boston, Massachusetts 02115
†Medical Genome Centre
John Curtin School of Medical Research
Canberra 2601
Australia

Summary

The role of complement in the maintenance of self-tolerance has been examined in two models: an immunoglobulin transgenic model of peripheral tolerance and a lupus-like murine model of CD95 (*Fas*) deficiency. We find that self-reactive B lymphocytes deficient in complement receptors CD21/CD35 or transferred into mice deficient in the complement protein C4 are not anergized by soluble self-antigen. In the second model, deficiency in CD21/CD35 or C4 combined with CD95 deficiency results in high titers of anti-nuclear antibodies leading to severe lupus-like disease. These findings suggest a novel role for the complement system in B cell tolerance and provide insight into the genetic association of complement deficiency with susceptibility to systemic lupus erythematosus.

Introduction

Systemic lupus erythematosus (SLE) is a common autoimmune disorder with a prevalence as high as 1 in 2500 individuals and a male to female ratio of 1 to 9 (Michet et al., 1985; Cotran et al., 1994). It is characterized by dysregulation of self-reactive B lymphocytes resulting in anti-nuclear and -dsDNA antibodies. Although unexplained, a major risk factor is deficiency in serum complement proteins C1, C2, or C4, as affected individuals (male and female equally) almost always develop SLE (Agnello, 1978; Walport and Morgan, 1991). Interestingly, deficiency in C3 is not a major risk factor. Recent results from biochemical studies (Fearon and Carter, 1995) and knockout mice (Carroll, 1998a) provide new insight into an essential role for the complement system in the activation and formation of memory B lymphocytes to thymus-dependent antigens (Pepys, 1972; Klaus et al., 1980). For example, mice deficient in either C3 ($C3^{null}$) or C4 ($C4^{null}$) (Fischer et al., 1996) (C4 is required in C3 activation) or complement receptors CD21 and CD35 ($Cr2^{null}$) (Ahearn et al., 1996; Croix et al., 1996; Molina et al., 1996) have an impaired humoral response to thymus-dependent antigens characterized by reduced number and size of germinal centers (GC). CD21 (molecular weight 150 kDa) and CD35 (molecular weight 190 kDa) are coexpressed on B cells and follicular dendritic cells (FDC); they are both encoded at the Cr2 locus, as CD21 is derived from CD35 by alternative splicing. Expression of CD21/CD35 is essential in at least two stages of B cell response to antigen: (1) retention within the lymphoid follicles and (2) GC survival (Fischer et al., 1998a). Survival of GC B cells is mediated by contact between C3d antigen–decorated FDC and the B cell coreceptor, a membrane complex of CD21/CD19/CD81 (Fischer et al., 1998a; Qin et al., 1998).

To determine if complement is also involved in negative selection of self-reactive B cells, two approaches were taken. In the first, $Cr2^{null}$ and $Cr2^{+}$ mice were bred with transgenic mice expressing transgenes encoding either soluble hen egg lysozyme (sHEL; ML5) or high-affinity HEL-specific immunoglobulin (Ig; strain MD4) (Goodnow et al., 1995). Breeding of the $Cr2^{null}$ or $Cr2^{+}$ Ig with sHEL mice provided a model to study the role of CD21/CD35 in B cell negative selection induced by a defined self-antigen, i.e., sHEL. In the double-transgenic mice, HEL-binding B cells are anergized upon contact with sHEL self-antigen within the bone marrow but escape to the periphery where they have a dramatically reduced half life (Fulcher and Basten, 1994) and are possibly eliminated by a *Fas*-dependent mechanism (Rathmell et al., 1995). In the periphery, anergic HEL-binding B lymphocytes exhibit a dramatic reduction in surface IgM expression (mIgM); ex vivo, they fail to respond to cross-linking of mIgM, even in the presence of T cell help (Cooke et al., 1994). By contrast, we find that $Cr2^{null}$ HEL-binding B lymphocytes accumulate in the spleen and lymph nodes of sHEL transgenic mice at normal frequencies, have an increased life span compared to $Cr2^{+}$ B cells, and respond to HEL self-antigen ex vivo by changes in intracellular calcium ion ($Ca^{2+}i$) and expression of the activation marker CD86 (B7–2). Importantly, the complement component C4 is critical for the maintenance of self-tolerance, as Ig transgenic B cells are not anergized in the presence of sHEL in $C4^{null}$ mice. In the second model, mice deficient in CD21/CD35, C4, or C3 were bred with CD95 (*Fas*) deficient mice (C57BL/6.*lpr/lpr*), which have a mild form of lupus-like disease (Theofilopoulos and Dixon, 1985). Mice bearing the combined deficiency of $Cr2^{null}$ or $C4^{null}$ and *lpr/lpr* developed a dramatic increase in anti-nuclear and -dsDNA antibodies, lymphadenopathy, and glomerulonephritis. By contrast, $C3^{null}$ *lpr/lpr* mice did not develop increased autoantibodies and disease was only modestly increased compared to complement-sufficient (C^{+}) *lpr/lpr* controls. These results support the hypothesis that complement is important in negative selection of self-reactive B cells and provide an explanation for the paradox in which complement deficiency is associated with autoimmunity.

‡To whom correspondence should be addressed (e-mail: mcarroll@rics.bwh.harvard.edu).
§These authors contributed equally to this work.

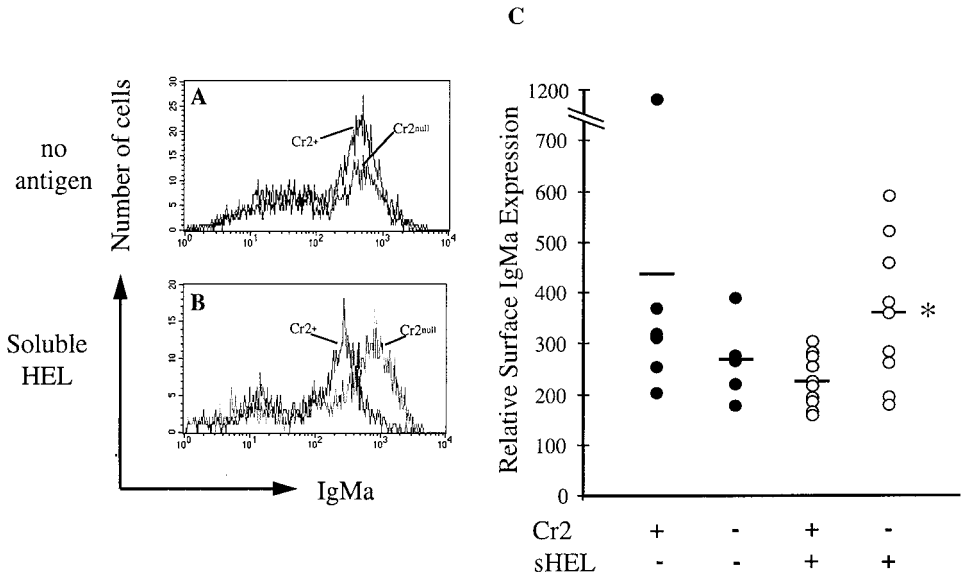

Figure 1. Surface IgMa Expression on Immature Bone Mature B Lymphocytes Is Not Reduced in $Cr2^{null}$ Mice in the Presence of sHEL

(A and B) Representative histograms of relative IgMa fluorescence of HEL-binding B cells of single- and double-transgenic $Cr2^{+}$ and $Cr2^{null}$ mice.

(C) Scatter plot summarizing results from at least three separate experiments. Results reveal that $Cr2^{null}$ HEL-binding B cells do not downregulate surface IgM in the presence of HEL self-antigen. Horizontal bars represent mean values.

Results

$Cr2^{null}$ HEL-Binding B Lymphocytes Mature and Accumulate Normally in the Presence of sHEL Self-Antigen

Encounter of immature (IgM^{+}, IgD^{-}, $CD23^{-}$) HEL-binding B cells with sHEL in the bone marrow results in downregulation of membrane IgM (mIgM) (Figures 1A–1C). By contrast, relative mIgM expression actually increases on $Cr2^{null}$ self-reactive B cells in the presence of self-antigen (225 ± 14 versus 380 ± 48 relative units of intensity, $p < 0.013$, respectively) (Figures 1A–1C). Three-color flow cytometry analysis confirmed that these were mIgD-negative immature B cells (data not shown). These results demonstrate that CD21/CD35 is involved in negative selection of B cells and suggest that complement is enhancing either B cell signaling or localization of HEL self-antigen to the bone marrow (BM).

Examination of splenocytes harvested from $Cr2^{+}$ single- and double-transgenic mice by three-color flow cytometry revealed a similar frequency of $IgMa^{+}B220^{+}$ B cells that bound HEL, approximately 81%–83% (Table 1). Interestingly, there was an approximate 2-fold difference in the number of $Cr2^{null}$ versus $Cr2^{+}$ HEL-binding B cells in double-transgenic mice ($52 \pm 13.9 \times 10^6$ versus $25 \pm 9.1 \times 10^6$, $p < 0.00001$ [Table 1]) despite a similar decrease in the level of expression of mIgM (data not shown). One possible explanation for the frequency increase is that the $Cr2^{null}$ Ig transgenic B cells are positively selected by sHEL self-antigen. Consistent with this interpretation is the finding of a modest increase in frequency of $Cr2^{null}$ HEL-binding B cells in double- compared to single-transgenic animals both in the bone marrow and spleen (Table 1). Histological analyses of cryosections of spleens harvested from $Cr2^{+}$ and $Cr2^{null}$ single- and double-transgenic mice confirmed that a higher frequency of $Cr2^{null}$ than $Cr2^{+}$ HEL-binding B cells were accumulating within the splenic follicles of double-transgenic mice (data not shown). These results suggest that in the absence of CD21/CD35 expression, self-reactive B cells are not anergized, but they continue to mature and accumulate within the peripheral compartment. This apparent break in tolerance is not due to an inherent difference in the level of self-antigen or antibody in the deficient mice, because sHEL and HEL-specific antibody in serum of $Cr2^{null}$ single-transgenic mice was comparable to that of $Cr2^{+}$ controls (data not shown).

sHEL self-antigen has a more striking effect on the accumulation of anergic B cells in the peripheral lymph nodes (LN), as the frequency of HEL-binding B cells was 3-fold less in double- than single-transgenic mice (Table 1). When all HEL-binding LN cells were analyzed for expression of CD23, a marker for mature B cells, an even greater difference was found (Table 1). A modest decrease in the frequency of $CD23^{+}$ $Cr2^{null}$ HEL-binding B cells was also observed in double-transgenic mice, but it was less than 2-fold (Table 1). Thus, a significantly higher number of mature $CD23^{+}$ $Cr2^{null}$ than $Cr2^{+}$ Ig transgenic B cells accumulates within the LNs ($2.0 \pm 1.2 \times 10^5$ versus $0.5 \pm 0.3 \times 10^5$, $p < 0.0007$). To examine turnover of the HEL-binding B cells in the absence or presence of HEL self-antigen, single- and double-transgenic mice were injected with BrdU each day for seven days; LN cells were harvested and analyzed by three-color flow cytometry. In this assay, nondividing cells fail to incorporate BrdU and can be distinguished from newly formed cells, which are BrdU positive. The frequency of BrdU-negative, $Cr2^{+}$ HEL-binding $B220^{+}$ cells in the lymph nodes of double-transgenic mice was reduced about one-third, i.e., 76% ± 3.3% to 55% ±

Table 1. Number of $Cr2^+$ and $Cr2^{null}$ HEL-Binding B Cells in the Bone Marrow, Spleen, and Lymph Nodes in the Presence or Absence of sHEL Antigen

		Bone Marrow		Spleen		Lymph Nodes		
Transgenic Mice	Antigen	Percent of HEL^+ B cells	Number of HEL^+ B cells ($\times 10^6$)	Percent of $B220^+$ Cells	Percent of HEL^+ B Cells	Number of HEL^+ B Cells ($\times 10^6$)	Percent of HEL^+ B Cells	Numberof HEL^+ B Cells ($\times 10^6$)	Number of $CD23^+$ HEL^+ B Cells ($\times 10^6$)
$Cr2^+$ (n = 6)	None	12% ± 4.4%	11.7 ± 4.3	30% ± 8.6%	25% ± 6.2%	23 ± 5.7	25% ± 6.0%	10.0 ± 1.9	5.3 ± 0.9
$Cr2^{null}$ (n = 5)	None	9% ± 2.6%	9.5 ± 4.5	29% ± 14.1%	23% ± 11.8%	26 ± 13.1	17% ± 5.4%	8.2 ± 2.4	3.8 ± 0.9
$Cr2^+$ (n = 12)	sHEL	13% ± 3.1%	11.0 ± 2.6	32% ± 10.4%	26% ± 9.8%	25 ± 9.1[a]	7% ± 5.4%	3.2 ± 1.4[b]	0.5 ± 0.3[c]
$Cr2^{null}$ (n = 15)	sHEL	14% ± 1.3%	11.5 ± 1.2	40% ± 9.3%	35% ± 9.5%	52 ± 13.9	18% ± 2.9%	8.2 ± 2.5	2.0 ± 1.2

Numbers and frequency (percent of total lymphocytes) of HEL-binding B cells were determined by flow cytometry as described in Experimental Procedures. Results represent mean values ± standard deviation. Number of $CD23^+$ HEL-binding B cells was calculated from gated cells (data not shown). Statistical comparisons are indicated by superscripts.

[a] Comparison of number of HEL-binding B cells in spleens of $Cr2^+$ and $Cr2^{null}$ double transgenic mice, $p < 0.00001$.

[b] Comparison of number of HEL-binding B cells in lymph nodes of $Cr2^+$ and $Cr2^{null}$ double-transgenic mice, $p < 0.000007$.

[c] Comparison of the number of $CD23^+$ HEL-binding B cells in lymph nodes of $CR2^+$ and $Cr2^{null}$ double-transgenic mice, $p < 0.001$.

12.5%, in the presence of sHEL self-antigen (Figures 2A and 2C). Thus, approximately half of the $Cr2^+$ HEL-binding B cells were replaced within the LN during the 7 day labeling period. This finding is consistent with that of Fulcher and Basten, who found 50% renewal rate of about 1 week for mature HEL-binding anergic B cells (Fulcher and Basten, 1994). Similarly, the total number of BrdU-negative, $Cr2^+$ HEL-binding $B220^+$ cells within the LNs was reduced approximately 3-fold ($7.5 \pm 1.62 \times 10^5$ versus $1.8 \pm 0.64 \times 10^5$, $p < 0.0001$) in the presence of sHEL antigen (Figure 2E). By contrast, very little difference was observed in the renewal of $Cr2^{null}$ HEL-binding $B220^+$ cells in the absence or presence of HEL self-antigen, i.e., 75% ± 4.7% BrdU negative versus 74% ± 4.8% BrdU negative, respectively (Figures 2B and 2D). Similarly, the total number of BrdU-negative $Cr2^{null}$ HEL-binding $B220^+$ cells did not change significantly in the presence of HEL self-antigen (Figure 2E). Thus, the presence of HEL self-antigen did not appear to alter significantly the turnover or total number of CD21/CD35-deficient Ig transgenic B cells within the LNs. The longer life span of the $Cr2^{null}$ HEL-binding B cells would explain their increased accumulation relative to the anergic $Cr2^+$ B cells.

$Cr2^{null}$ HEL-Binding B Lymphocytes Respond to Antigen Ex Vivo

In the double-transgenic model, anergic B cells are characterized by their failure to respond to cross-linking of mIgM in vitro as determined by change in intracellular $Ca^{2+}i$ and expression of the activation marker CD86 (B7–2) (Cooke et al., 1994). To test if the $Cr2^{null}$ HEL-binding B cells were anergized by sHEL self-antigen, $Cr2^+$ and $Cr2^{null}$ HEL-binding B cells were harvested from the spleens of single- and double-transgenic mice and characterized in vitro for their response to HEL. Splenocytes were cultured with 100 ng/ml of HEL antigen overnight, and cells were examined the next day by three-color flow cytometry for surface expression of the activation markers CD86, HEL, and B220. As expected, very few anergic $Cr2^+$ Ig transgenic B cells responded to HEL (Figures 3A, 3C, and 3I). This lack of response to HEL is not explained by the reduced number of HEL-binding B cells, as their frequency is similar in the $Cr2^+$ single- and double-transgenic mice (Table 1). By contrast, the frequency of $CD86^+$ $Cr2^{null}$ $B220^+$ cells isolated from single and double transgenic mice was similar (Figures 3B, 3D, and 3I). The response to HEL is specific in this assay, as very few $B220^+$ cells harvested from spleens of either group of double-transgenic mice expressed CD86 before culture (Figures 3E and 3F), and wild-type (nontransgenic) $B220^+$ splenic B cells do not show a change in CD86 expression after overnight culture either without or with HEL stimulation (Figures 3G and 3H). To examine signal transduction, splenocytes isolated from single- and double-transgenic mice were loaded with a calcium-sensitive dye (fluo-3), exposed to lysozyme (100 ng/ml), and $B220^+$ cells were analyzed by two-color flow cytometry for release of $Ca^{2+}i$. As reported by Cooke et al. (1994), cross-linking of mIgM with antigen failed to induce significant $Ca^{2+}i$ release in anergic $Cr2^+$ HEL-binding cells (Figures 3J, 3L, and 3N). By contrast, only

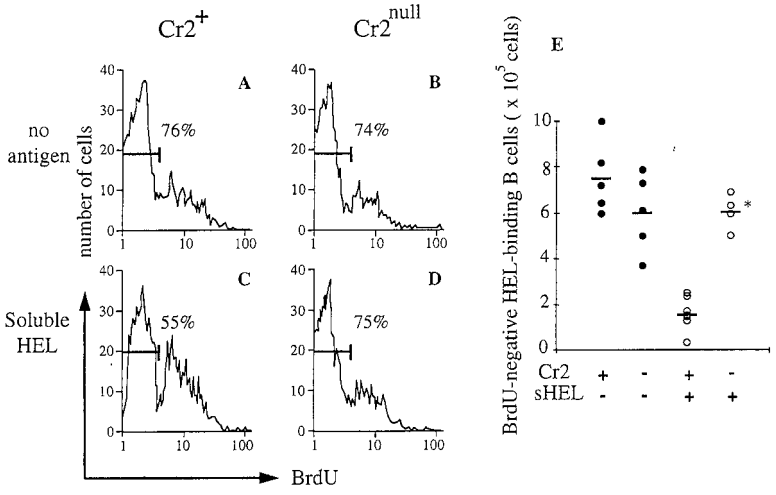

Figure 2. Life Span of Cr2^{null} HEL-Binding B Cells Is Not Reduced by Presence of HEL Self-Antigen

(A–D) Representative histograms of relative BrdU fluorescence of HEL-binding B cells of single- and double-transgenic Cr2^{+} and Cr2^{null} mice.

(E) Scatter plot represents total numbers of BrdU-negative HEL-binding B cells from five lymph nodes per mouse. Horizontal bar for each group of mice represents the mean number of total cells. Results are from at least three separate experiments. The asterisk indicates statistical significance ($p < 0.00008$) comparing number of BrdU-negative HEL-binding B cells harvested from LNs of Cr2^{+} and Cr2^{null} double-transgenic mice.

a relatively modest impairment in Ca^{2+}i mobilization was observed in the treated Cr2^{null} Ig transgenic B cells harvested from double-transgenic mice (Figures 3K, 3M, and 3N). The frequency of Cr2^{null} versus Cr2^{+} HEL-binding cells that mobilized Ca^{2+}i during the 60–120 s time interval was significantly greater (12% ± 3.9% versus 6% ± 1.3%, $p < 0.01$) (Figures 3L–3N). Thus, sHEL self-antigen fails to completely anergize Cr2^{null} HEL-binding B cells, as they mobilize Ca^{2+}i and express the activation marker CD86 in response to antigenic stimulation ex vivo. However, serum anti-HEL levels were undetectable as found for the anergic Cr2^{null} double trasngenic mice (data not shown).

HEL-Binding B Lymphocytes Are Not Anergized by sHEL Self-Antigen in Mice Deficient in C4

To examine the importance of the complement proteins C4 and C3 in negative selection of HEL-binding B cells in the transgenic model, chimeric mice were constructed by engrafting BM harvested from C^{+} HEL-specific Ig transgenic mice into either C4^{null} , C3^{null}, or C^{+} non- or sHEL-transgenic recipients. This approach provides an alternative to breeding of transgenic mice as HEL-binding transgenic B cells are similarly anergized by sHEL self-antigen in chimeric mice as described above (Goodnow et al., 1995). A potential limitation with the chimeras is that bone marrow–derived cells from the C^{+} donor could be sources of C4 or C3 protein in lymphoid tissues. Extrahepatic synthesis of C4 or C3 (Fischer et al., 1998b) is not constitutive and C4 or C3 protein was not detected in the serum of the respective chimeras (data not shown).

Characterization of BM chimeras 4–6 weeks after transplant by three-color flow cytometry confirmed a frequency of HEL-binding B cells in single-transgenic chimeras similar to that found in the germline mice described above (Table 1), i.e., approximately 80%–85% of B220^{+} cells (Table 2). Flow cytometry analyses of immature HEL-binding B cells in the C4^{null} and C^{+} sHEL/HEL Ig transgenic chimeras indicated a relative increase in the level of mIgM on the surface of B cells harvested from the C4^{null} mice consistent with that observed with Cr2^{null} double-transgenic mice (data not shown). Interestingly, in the presence of sHEL self-antigen, the number of HEL-binding B cells in spleens of C^{+} and C3^{null} Ig transgenic chimeras was reduced by two-thirds and one-third, respectively ($32 \pm 5.3 \times 10^6$ versus $10 \pm 2.8 \times 10^6$, respectively [$n \geq 9$]; and $34 \pm 6.4 \times 10^6$ versus $18 \pm 5.0 \times 10^6$, respectively [$n \geq 4$]). While this reduction is more pronounced than that observed in double-transgenic mice produced by breeding (Table 1), it is comparable to that reported by Fulcher and Basten, who examined older chimeric animals (3–4 months) as used in this study (Fulcher and Basten, 1994). By contrast, a similar number of HEL-binding B cells was found in spleens of single- and double-transgenic C4^{null} chimeras ($26 \pm 5.2 \times 10^6$ versus $27 \pm 5.8 \times 10^6$, respectively [$n \geq 9$]). Thus, significantly fewer HEL-binding B cells were found in spleens of C^{+} and C3^{null} than in C4^{null} double-transgenic chimeras ($10 \pm 2.8 \times 10^6$ or $18 \pm 5.0 \times 10^6$ versus $27 \pm 5.8 \times 10^6$; $p < 0.00001$ and < 0.003, respectively [$n \geq 8$]). Consistent with these findings, histological analysis revealed a reduction in the frequency of HEL-binding B cells in the splenic follicules of double-transgenic C^{+} or C3^{null} chimeras but not in C4^{null} chimeras (data not shown).

Analysis of mature CD23^{+} HEL-binding B cells in the peripheral LNs revealed a striking reduction in the number of transgenic B cells in the C^{+} and C3^{null} double-transgenic mice compared to C4^{null} double-transgenic chimeras. Approximately 11-fold fewer HEL-binding B cells mature and reach the LNs in the C^{+} compared to C4^{null} double-transgenic animals ($0.5 \pm 0.3 \times 10^4$ versus $5.7 \pm 1.8 \times 10^4$, $p < 0.00003$ [$n \geq 4$]). Three-fold fewer mature transgenic B cells were identified within peripheral LNs of C3^{null} compared to C4^{null} double-transgenic mice ($2.1 \pm 1.1 \times 10^4$ versus $5.7 \pm 1.8 \times 10^4$, $p < 0.0003$ [$n \geq 8$]). Thus, as found in the spleen, the number of HEL-binding B cells is intermediate in C3^{null} double-transgenic chimeras compared to C^{+} and C4^{null} animals. The finding that deficiency in C4 alters negative selection of immature self-reactive B cells is consistent with results described above with the Cr2^{null} double-transgenic mice. Interestingly, deficiency in serum C3 has less of an effect.

C4^{null} HEL-Binding B Lymphocytes Respond to Antigen Ex Vivo

As a further measure of anergy, splenocytes isolated from C^{+}, C4^{null}, and C3^{null} single- and double-transgenic chimeras were characterized in vitro by three-color flow

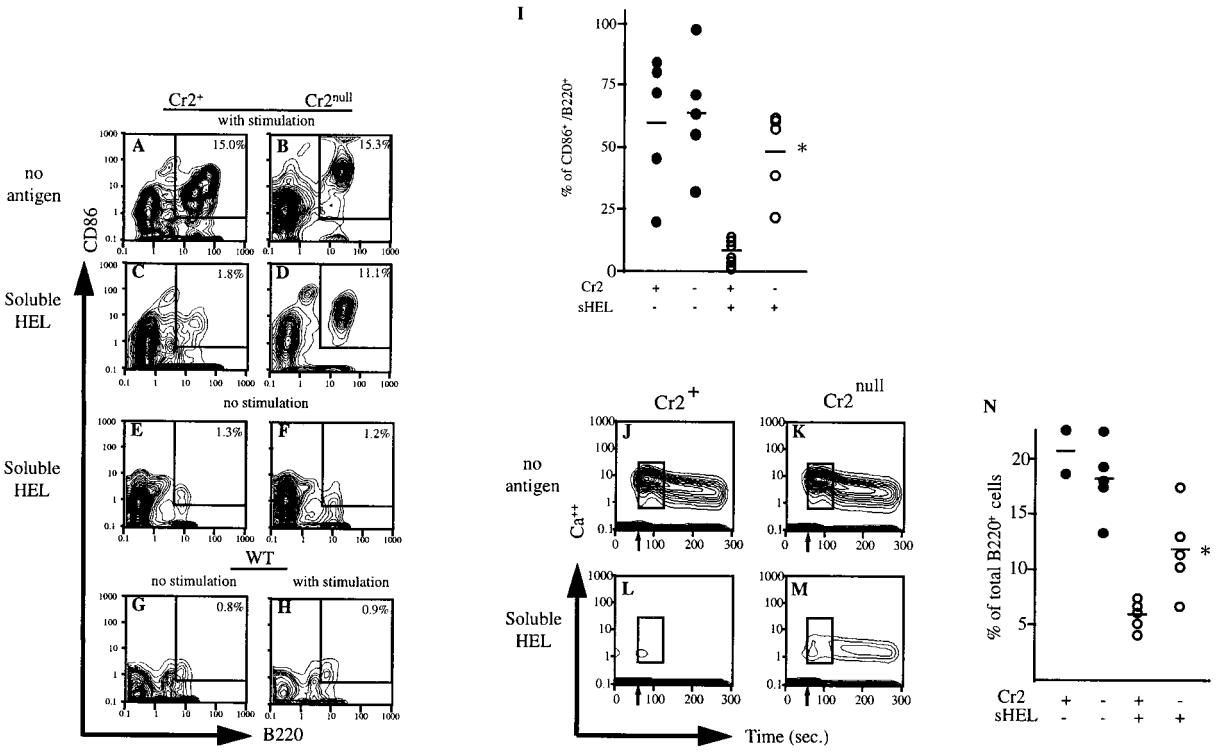

Figure 3. $Cr2^{null}$ HEL-Binding B Cells Harvested from Double-Transgenic Mice Are Not Anergic Based on Expression of Activation Marker CD86 and Mobilization of Intracellular Ca^{2+}i in Response to Antigen Stimulation Ex Vivo

(A–D) Representative flow cytometry results of HEL-binding splenocytes following overnight culture with 100 ng/ml of HEL and staining with MAb specific for B220 and CD86 (B7–2). HEL^+, $B220^+$ cells harvested from $Cr2^+$ double-transgenic mice have an impaired response to HEL self-antigen as expected from previous studies (Cooke et al., 1994). By contrast, only a modest decrease in responsiveness to antigen stimulation was observed in $Cr2^{null}$ cells isolated from double-transgenic mice. Number in upper right quadrant represents percent of total lymphocytes gated that are double positive for CD86 and B220. (E and F) Response to culture with antigen was confirmed as specific, as very few $B220^+$ cells isolated from the two groups of double-transgenic mice expressed CD86 ex vivo prior to culture with HEL, and (G and H) wild-type spleen cells express background levels of CD86 ex vivo irrespective of culture without or with HEL. (I) Scatter plot summarizing results from three separate experiments demonstrating a significant difference ($p < 0.004$, indicated by asterisk) in the frequency of $CD86^+B220^+$ cells following overnight culture with HEL isolated from $Cr2^{null}$ compared to $Cr2^+$ double-transgenic mice. Horizontal bars represent means for each group of mice analyzed. Results represent the percent of total $B220^+$ cells that were positive for CD86 based on gate settings shown in (A–D). (J–M) Representative flow cytometry results of splenocytes showing mobilization of Ca^{2+}i following brief stimulation with 100 ng/ml of HEL. Results demonstrate that splenocytes harvested from $Cr2^{null}$ double transgenic mice retain responsiveness ex vivo to HEL antigen, while $Cr2^+$ self-reactive cells are anergic as expected. Gated cells represent Ca^{2+}i measured for a time period of 60–120 s. (N) Scatter plot represents summary of results from two separate experiments for analysis of frequency of $B220^+$ cells, which mobilize Ca^{2+}i over the time period of 60–120 s. The bar represents the mean and the asterisk indicates statistical significance ($p < 0.01$; comparison of cells harvested from $Cr2^+$ and $Cr2^{null}$ double-transgenic mice).

cytometry for expression of the activation marker CD86 as described above. Following overnight culture with HEL antigen, a relatively low frequency of $CD86^+B220^+$ B cells was observed in C^+ and $C3^{null}$ double- compared to single-transgenic chimeric mice (7% ± 4.3% and 4% ± 3% versus 77% ± 11.3% and 78% ± 3.9%, $p <$ 0.00003, respectively) (Figures 4A–4F and 4J). By contrast, a similar frequency of $CD86^+B220^+$ responding cells was observed on comparison of single- and double-transgenic $C4^{null}$ chimeras (Figures 4B, 4E, and 4J). Thus, a significantly higher proportion of the HEL-binding B cells isolated from $C4^{null}$ than C^+ or $C3^{null}$ chimeric mice expressed CD86 in response to HEL antigen ex vivo, i.e., 49% ± 19% versus 7% ± 4.3% or 4% ± 3% ($p <$ 0.00002 or 0.00001, respectively) (Figure 4J). Likewise, both C^+ and $C3^{null}$ chimeric double-transgenic mice had significantly impaired Ca^{2+}i mobilization compared to $C4^{null}$ chimeric double transgenics (2.4% ± 1.1% or 1.3% ± 0.45% versus 8.5% ± 2.6%, respectively; $n \geq 5$; $p <$ 0.0001 or 0.00007, respectively) when incubated with HEL antigen. Although, the frequency of HEL-binding cells is less in the spleens of C^+ and $C3^{null}$ chimeras than $C4^{null}$ double-transgenic chimeras, this difference would not explain the overall increase in frequency of $CD86^+B220^+$ cells in the later group or the increased Ca^{2+}i mobilization by the $C4^{null}$ chimeric mice. Thus, C4 is important in induction of anergy in the double-transgenic HEL model, but serum C3 does not play a role in B cell tolerance.

Autoimmune Disease Is Increased in *lpr/lpr* Mice Totally Deficient in either C4 or CD21/CD35 but Not C3

The results from the well-characterized, high-affinity immunoglobulin transgenic model provide strong evidence for a role for complement in negative selection

Table 2. Number of HEL-Binding B Cells Harvested from C^+, $C3^{null}$, and $C4^{null}$ Single- and Double-Transgenic Mice in the Bone Marrow, Spleen, and Lymph Nodes

		Bone Marrow		Spleen			Lymph Nodes		
Donor aHEL Transgenic Mice	Recipient	Percent of HEL^+ B Cells	Number of HEL^+ B Cells ($\times 10^5$)	Percent of $B220^+$ Cells	Percent of HEL^+ B Cells	Number of HEL^+ B Cells ($\times 10^6$)	Percent of HEL^+ B Cells	Number of HEL^+ B Cells ($\times 10^4$)	Number of $CD23^+$ HEL^+ B Cells ($\times 10^4$)
C^+ (n = 9)*	C+	7.5% ± 1.4%	7.4 ± 1.3	33% ± 4.4%	29% ± 4.7%	32 ± 5.3	5.1% ± 1.8%	5.6 ± 1.9	4.1 ± 1.4
C^+ (n = 9)*	$C4^{null}$	6.1% ± 0.6%	6.5 ± 1.3	34% ± 7.2%	30% ± 6.0%	26 ± 5.2	6.6% ± 2.3%	7.2 ± 2.2	4.3 ± 1.3
C^+ (n = 4)	$C3^{null}$	5.1% ± 0.8%	6.2 ± 1.5	32% ± 2.7%	29% ± 5.4%	34 ± 6.4	6.2% ± 1.6%	7.7 ± 1.9	6.9 ± 1.8
C^+ (n = 10)*	C^+ sHEL	7.1% ± 0.6%	8.0 ± 0.6	23% ± 4.9%	16% ± 4.3%	10 ± 2.8[a]	1.8% ± 0.9%	2.3 ± 1.3[b]	0.5 ± 0.3[b]
C^+ (n = 10)**	$C4^{null}$ sHEL	8.5% ± 1.3%	8.5 ± 1.1	36% ± 6.0%	28% ± 6.0%	27 ± 5.8	6.1% ± 1.9%	7.8 ± 2.5	5.7 ± 1.8
C^+ (n = 8)	$C3^{null}$ sHEL	7.4% ± 1.1%	8.5 ± 1.2	23% ± 2.0%	19% ± 5.2%	18 ± 5.0[c]	3.0% ± 1.6%	3.4 ± 1.8[d]	2.1 ± 1.1[d]

Numbers and frequency (percent of total lymphocytes) of HEL-binding B cells were determined by flow cytometry as described in Experimental Procedures. Results represent mean values ± standard deviation. Number of $CD23^+$ HEL-binding B cells was calculated from gated cells (data not shown). Statistical comparisons are indicated by superscripts.

[a,c] Comparison of number of HEL-binding B cells in spleens of $C4^{null}$ versus C^+ or $C3^{null}$ double-transgenic mice, $p<0.000001$ and $p<0.003$, respectively.

[b,d] Comparison of total number of HEL-binding B cells or $CD23^+$ HEL-binding B cells in lymph nodes of $C4^{null}$ versus C^+ or $C3^{null}$ double-transgenic mice, $p < 0.0003$ or $p < 0.0007$, respectively. One asterisk indicates n = 4 for BM and LNs; two asterisks indicate n = 8 for BM and LNs.

of HEL-binding B cells; however, they raise the question of whether complement is also important for maintenance of tolerance to natural self-antigens, as is commonly found in SLE (e.g., nuclear antigens). To address this question, we examined a murine model of lupus in an *lpr/lpr* strain of mice (Theofilopoulos and Dixon, 1985). These lupus-prone mice have a natural deficiency in CD95 (*Fas*) that leads to disease presumably due to the failure to eliminate self-reactive T and B lymphocytes from the periphery (Takahashi et al., 1994). Severity of disease is strain dependent, and in the C57BL/6 background *Fas* deficiency results in a mild form of the disease in which the mice have a normal life span and do not develop glomerulonephritis. To examine the role of complement in the mouse SLE model, $Cr2^{null}$, $C4^{null}$, or $C3^{null}$ mice were bred to C57BL/6.*lpr/lpr* mice and characterized for severity of disease.

A common phenotype of *Fas* deficiency is splenomegaly and lymphadenopathy presumably due to the expansion of a novel population of CD4- and CD8-negative but $B220^+CD3^+$ T lymphocytes. Complement-sufficient *lpr/lpr* mice (C^+*lpr/lpr*) on the mixed 129 × C57BL/6 background have slight splenomegaly and lymphadenopathy at 13 and 17 weeks of age consistent with their mild disease (Figures 5A and 5B). By contrast, *lpr/lpr* mice deficient in either CD21/CD35 or C4 but not C3 have significantly increased mass of their cervical lymph nodes at both 13 and 17 weeks of age; $Cr2^{null}$ *lpr/lpr* mice also have significant splenomegaly (Figures 5A and 5B). One explanation for the increased sizes in spleen and lymph nodes is that the $C4^{null}$ or $Cr2^{null}$ *lpr/lpr* mice develop a more vigorous expansion of the double-negative $CD3^+$ T cell population, possibly resulting from increased expansion of self-reactive B cells. This explanation would be consistent with the observation that lymphoproliferation and disease is reduced in B cell–deficient MRL-*lpr/lpr* mice (Chan and Shlomchik, 1998).

A hallmark of lupus is autoantibodies against nuclear antigens such as ribonuclear proteins, histones, and double-strand DNA (dsDNA) (Cotran et al., 1994). To determine if deficiency in complement affects production of anti-nuclear or -dsDNA antibodies, sera were collected from the four groups of *lpr/lpr* mice at 10, 13, and 17 weeks of age and assayed by immunofluorescence and ELISA, respectively. Analysis of anti-nuclear antibody (ANA) titers for the three groups of mice revealed a striking increase in total IgG antibodies in the C4- and CD21/CD35-deficient mice, while only background levels of autoantibody were found for the C^+ *lpr/lpr* and $C3^{null}$ *lpr/lpr* mice (Figure 5C). Consistent with increased ANA, both $C4^{null}$ and $Cr2^{null}$ *lpr/lpr* mice expressed significantly higher levels of anti-dsDNA antibodies (Figure 5D). The finding of high titers of anti-nuclear and -dsDNA antibodies supports our hypothesis that SLE-related self-reactive B cells are not eliminated in the absence of C4 or CD21/CD35 and that in the presence of T cell help and self-antigen they secrete autoantibody. A major pathogenic consequence of circulating autoantibodies in SLE is deposition of immune complexes within the kidney leading to glomerulonephritis, which can be fatal (Cotran et al., 1994). Examination of cryosections of kidneys by immunofluorescence revealed increased deposition of IgG in the glomeruli of

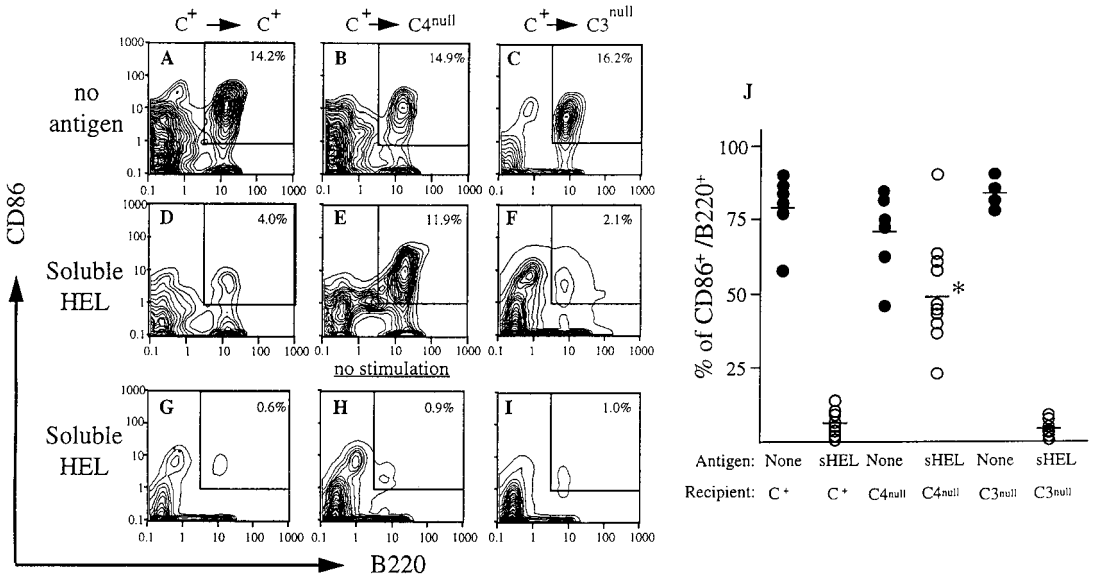

Figure 4. HEL-Binding B Cells Harvested from C4^{null} but Not C3^{null} Chimeric-Double Transgenic Mice Respond to Antigen Ex Vivo and Express the Activation Marker CD86 (B7–2)

(A–F) Representative flow diagram indicating frequency of B220^{+} splenocytes harvested from C^{+}, C4^{null}, or C3^{null} single- and double-transgenic mice, which express CD86 in response to antigen stimulation in vitro. Results reveal that HEL-binding B cells harvested from C4^{null} double-transgenic mice are responsive to HEL in vitro and therefore do not appear to be anergic. By contrast, HEL-binding B cells harvested from C^{+} or C3^{null} chimeric double-transgenic mice are anergized. Numbers in upper right quadrants indicates mean percent of B220/CD86 double-positive cells out of total lymphocytes gated.

(G–I) Representative flow diagrams of HEL-binding B cells harvested from the three groups of double-transgenic mice and analyzed directly without prior stimulation. Results reveal that despite presence of sHEL antigen in vivo the B cells do not appear to be activated.

(J) Scatter plot summarizing results from three separate experiments comparing frequency of CD86^{+} cells out of total B220^{+} splenocytes gated. Horizontal bars indicate mean values. Asterisk indicates statistical significance ($p < 0.002$) comparing frequency of CD86^{+} splenocytes harvested from C^{+} or C3^{null} with C4^{null} double-transgenic mice.

Cr2^{null} or C4^{null} *lpr/lpr* compared to C^{+} *lpr/lpr* mice, i.e., of 100 glomeruli counted, 92%, 58%, and 15% were positive, respectively; $n \geq 3$ mice per group (Figures 5L, 5J, and 5F). A modest increase in IgG deposits was also observed in glomeruli of C3^{null} *lpr/lpr* mice despite their relatively low levels of anti-nuclear and -dsDNA antibody (Figure 5H). C3 deposition was also identified in the glomeruli of C4 and CD21/CD35-deficient but not complement-sufficient or C3^{null} *lpr/lpr* mice (data not shown). Consistent with deposition of IgG and C3, increased hypercellularity and glomerular enlargement was found in the glomeruli of Cr2^{null} and C4^{null} but not C^{+} *lpr/lpr* mice (mean scores of 2–3, 1–2, and 1 for Cr2^{null}, C4^{null}, and C^{+} *lpr/lpr*, respectively, were assigned based on criteria in Experimental Procedures) (Figures 5K, 5I, and 5E). Thus, C4^{null} or Cr2^{null} *lpr/lpr* mice have increased SLE-like autoantibodies and deposits of immune complexes within the glomeruli that can lead to severe glomerulonephritis (Figures 5E–5L). Similar to human lupus, C3^{null} *lpr/lpr* mice did not have elevated autoantibody levels, and only a modest increase in IgG deposits was observed in the kidneys.

Discussion

Our findings describe a novel role for complement in maintenance of B cell tolerance to soluble self-antigens. Using the HEL-Ig/sHEL double-transgenic model, we found that induction of anergy in HEL-binding self-reactive B cells required expression of complement receptors CD21/CD35. Receptor-deficient mice showed a dramatic reduction in tolerance as the HEL-binding B cells matured and accumulated within the peripheral lymphoid compartment at similar frequencies to C^{+} single-transgenic mice (Figure 2; Table 1). Cr2^{null} transgenic B cells were not anergized by the presence of self-antigen when analyzed in vitro, based on both mobilization of Ca^{2+}i and expression of CD86 (Figure 3). Deficiency in complement C4 leads to a similar loss of tolerance in the HEL model, and the results parallel those observed with Cr2^{null} double transgenics (Figure 4; Table 2). Despite the reduction in anergy, HEL-specific antibody was not detected in the serum of the Cr2^{null} or C4^{null} double-transgenic mice (data not shown). Interestingly, deficiency in serum C3 protein had less of an effect on tolerance. For example, the reduction in number of peripheral transgenic B cells in C3^{null} chimeric double transgenics was intermediate between C^{+} and C4^{null} chimeric mice. However, HEL-binding B cells harvested from the C3^{null} double transgenics remained unresponsive to antigen stimulation in vitro. A potential limitation with the transgenic model is that the HEL self-antigen binds with an unusually high affinity. Therefore, to extend our findings in a more natural model, we crossed the three C-deficient strains to a strain that develops a mild lupus-like disease. In this model, *Fas* deficiency would not be expected to alter the proposed role of C4

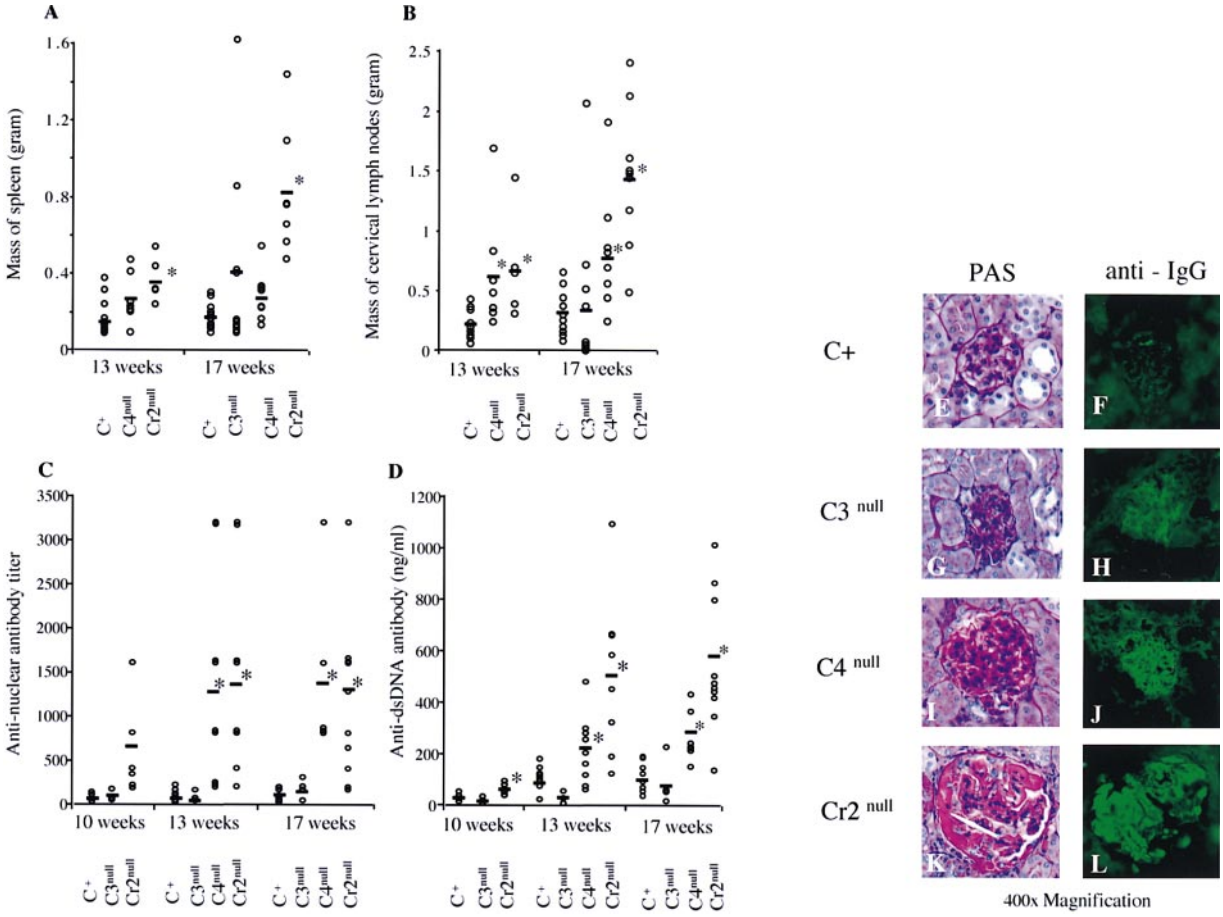

Figure 5. Deficiency in Complement CD21/CD35 or C4 Results in Increased Lymphadenopathy, which Correlates with Increased Anti-Nuclear and dsDNA Antibodies, Leading to Immune Deposits and Glomerulonephritis in *lpr/lpr* Mice

(A and B) Results demonstrate significant increase in mass of cervical lymph nodes at 13 and 17 weeks in $Cr2^{null}$ and $C4^{null}$ compared to $C3^{null}$ and C^+ *lpr/lpr* mice; $Cr2^{null}$ *lpr/lpr* mice also had significant splenomegaly at 13 and 17 weeks relative to C^+ *lpr/lpr*. Scatter blot indicates mass of spleens and cervical lymph nodes for individual mice at 13 and 17 weeks of age. Bars represent mean mass. Results are based on at least three experiments with female mice. Asterisk represents statistical significance $p < 0.02$.

(C) Sera from C^+ *lpr/lpr*, $C4^{null}$ *lpr/lpr*, $C3^{null}$ *lpr/lpr*, and $Cr2^{null}$ *lpr/lpr* were harvested at indicated times, and IgG titers were determined by indirect immunofluorescence. Results reveal significantly higher titers of anti-nuclear antibody in $Cr2^{null}$ *lpr/lpr* mice at all three time points. $C4^{null}$ *lpr/lpr* mice, which were not tested at 10 weeks, had high titers similar to $Cr2^{null}$ *lpr/lpr* mice at 13 and 17 weeks. Significantly, $C3^{null}$ *lpr/lpr* mice did not have autoantibody titers elevated over C^+*lpr/lpr* controls.

(D) Sera harvested as described above in (A) were analyzed by ELISA for IgG_{2B} binding to dsDNA. Results demonstrate that $C4^{null}$ and $Cr2^{null}$ *lpr/lpr* mice have significantly higher anti-dsDNA titers than complement-sufficient controls or $C3^{null}$ *lpr/lpr* at 13 and 17 weeks of age. IgG_{2B} isotype represents the major class of IgG in the anti-dsDNA antibody among the three groups of mice (data not shown). Results are from at least three experiments with female mice. Each open circle represents an individual mouse. Asterisk indicates significance $p < 0.04$.

(E–L) Histological analyses of kidneys harvested from 4 groups of *lpr/lpr* mice: C^+, $C3^{null}$, $C4^{null}$, and $Cr2^{null}$. (F, H, J, and L) Indirect immunofluorescent analyses of cryosections reveal increased frequency of IgG deposits in glomeruli of $Cr2^{null}$ (92% of glomeruli) (L) and $C4^{null}$ (56% of glomeruli) (J) mice but only intermediate to background levels in $C3^{null}$ (36% of glomeruli) (H) or C^+ (12% of glomeruli) *lpr/lpr* mice (F). (E, G, I, and K) Histological analysis of PAS-stained sections of kidneys indicates development of glomerulonephritis as characterized by hypercellularity and glomerular thickening. $Cr2^{null}$ *lpr/lpr* mice have more severe disease than $C4^{null}$, $C3^{null}$, and C^+ *lpr/lpr* mice with mean scores of 2–3, 2, 1–2 and 1, respectively ($n \geq 4$ mice per group; see Experimental Procedures for scoring criteria). Note hyaline deposition and near end-stage sclerotic lesion in $Cr2^{null}$ glomerulus (K). Magnification, 400×.

or CD35 in induction of anergy, as *Fas* is thought to be important in elimination, not induction, of anergic cells (Rathmell et al., 1995). Importantly, deficiency in either CD21/CD35 or C4 resulted in increased lymphadenopathy, anti-nuclear and-dsDNA autoantibodies that correlated with increased renal deposits of IgG, and glomerulonephritis. These findings demonstrate that complement C4 and its receptors CD21/CD35 are important in maintenance of B cell tolerance against natural self-antigens. Despite a role for complement in the humoral response to T-dependent antigens, this critical role is overcome in the production of autoantibody in the $C4^{null}$ and $Cr2^{null}$ *Fas*-deficient mice. The observation that C3 deficiency had no obvious affect on autoantibody levels is similar to that found in human lupus.

The fate of immature self-reactive B lymphocytes within the bone marrow and peripheral lymphoid compartment is largely determined by the strength of signal

mediated by the B cell receptor (BCR) in response to cross-linking by antigen (Nemazee and Burki, 1989; Erikson et al., 1991; Goodnow, 1996). For example, in double-transgenic models in which the self-antigen is expressed on the membrane, self-reactive B cells are clonaly deleted in the BM (Nemazee and Burki, 1989; Goodnow, 1996). Thus, the concentration and affinity of antigen and regulation of BCR signaling by molecules such as CD19 (Inaoki et al., 1997), CD45 (Cyster et al., 1996), or protein tyrosine phosphatase 1C (SHP-1) (Cyster and Goodnow, 1995) can dramatically affect negative selection of self-reactive B cells. It is not clear how soluble self-antigens are made available in negative selection within the primary and secondary lymphoid compartments. An explanation for our findings is that complement enhances localization of soluble self-antigens to the primary and secondary lymphoid compartments. Coreceptor signaling via C3d-coated self-antigens binding to CD21/CD19/Tapa-1 on B cells is not likely to play an important role based on our results with the $C3^{null}$ mice. We propose that complement enhances B cell tolerance against highly conserved soluble antigens such as DNA and nuclear proteins (Carroll, 1998b). Recent studies by Rosen and colleagues reported that dying cells release surface blebs that contain these types of antigens (Casciola-Rosen et al., 1994). Mice deficient in C1q spontaneously develop anti-nuclear autoantibodies and glomerulonephritis, which correlates with an accumulation of apoptotic bodies in the kidneys (Botto et al., 1998). Recognition of nuclear antigens by natural antibody (IgM) (Coutinho et al., 1995) or directly by C1q (Korb and Ahearn, 1997) would activate the early classical pathway (C1-C4) leading to covalent attachment of C4b. Uptake of C4b-coated self-antigens by CD35-bearing cells within the BM and secondary lymphoid compartment would provide a mechanism for concentration of self-antigens and insure contact with immature self-reactive B cells. An alternative hypothesis is that deficiency in C1-C4 results in accumulation of aggregates of self-antigen that activate C3 (by alternative pathway) and break B cell tolerance via coreceptor signaling. This hypothesis seems unlikely to explain the impaired tolerance in the HEL model, since $Cr2^{null}$ HEL-binding B cells in double-transgenic mice do not appear activated in vivo, i.e., high frequency of BrdU-negative mature B cells and low frequency of expression of the activation marker CD86 expressed by unstimulated transgenic B cells. However, it is possible that both mechanisms are involved in a complex autoimmune disease such as lupus.

In summary, examination of complement deficiency in two well-defined murine models of peripheral tolerance reveals that complement C4 protein and the receptors CD21/CD35 are involved in negative selection of self-reactive B lymphocytes. These results not only provide insight into the targeting of self antigens for maintenance of B cell tolerance; but provide an explanation for the long-standing paradox of how an immune deficiency predisposes to SLE.

Experimental Procedures

Mice

Mice transgenic for soluble hen egg lysozyme (sHEL) (ML5) were bred with C^+, $Cr2^{null}$, $C3^{null}$, and $C4^{null}$ mice (Ahearn et al., 1996; Fischer et al., 1996) (mixed 129/C57BL/6 backgrounds). Mice expressing transgenes encoding IgM/IgD heavy and light chain, high-affinity HEL-specific antibody (MD4) (Goodnow et al., 1995) (both on C57BL/6 background) were crossed with C^+ or $Cr2^{null}$ mice. Double-transgenic C^+ or $Cr2^{null}$ sHEL/anti-HEL mice were obtained by breeding the respective single transgenics. Offspring were genotyped by PCR using J_H-specific primers (IgH_{F2} and IgH_{R1}) to detect HEL Ig transgenics and HEL3F and HEL4R primers were used to detect sHEL transgenics. Germline transgenic mice used for experiments were 5–8 weeks old. $C3^{null}$, $C4^{null}$, and C^+ control chimeric mice were constructed as described (Fischer et al., 1996). Briefly, 1×10^7 bone marrow cells (harvested from C^+ HEL-specific Ig transgenic mice) were injected intravenously into $C3^{null}$, $C4^{null}$, or C^+ mice, which had been tested previously for sHEL expression (irradiated twice with 450 rads each at 3 hr apart) 1 hr after second irradiation. Chimeras were rested for approximately 30 days before use. Serum levels of sHEL and anti-HEL antibody were assayed by ELISA as described (Goodnow et al., 1989). Briefly, sHEL levels were determined by adding dilutions of serum to 96-well plates coated with HEL-specific MAb HyHEL-5 (5 μg/ml), followed by treatment with a second HEL-specific MAb HyHEL-9 conjugated with biotin. HEL binding was detected by adding strepavidin-alkaline phosphatase and development with a color reaction. Similarly, serum anti-HEL levels were assayed in single- and double-transgenic mice by adding dilutions of sera to 96-well plates coated with HEL antigen (50 μg/ml) followed by a goat anti-mouse IgM antibody conjugated with alkaline-phosphatase (Southern Biotechnologies, Birmingham, AL) and color development. C57BL/6 *lpr/lpr* mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were bred with our $Cr2^{null}$, $C3^{null}$, or $C4^{null}$ mice. Cr2, C3, or C4 typing was performed as described, and *lpr/lpr* homozygosity was confirmed with PCR as described (Erikson et al., 1991). All mice were homozygous for *lpr* and either homozygous $Cr2^{null}$, $C3^{null}$, or $C4^{null}$. C^+ controls represent homozygous $+/+$ offspring from mating $+/-$ mice. At age 10, 13, or 17 weeks, mice were bled and sacrificed, and spleen, cervical lymph nodes, and kidneys were harvested and weighed.

Flow Cytometry Staining and Analysis

Bone marrow, splenic, or lymph node mononuclear cells were prepared on a buoyant density gradient before staining and three-color analysis was conducted on a FACSCalibur as described (Fischer et al., 1998a). Antibodies used in this study included fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated rat anti-mouse isotype-control antibodies (R35–95, R35–38), anti-CD45R (B220), anti-CD23 (B3B4), anti-CD86 (GL1), and anti-IgMa (DS-1) monoclonal antibodies (all antibodies are from PharMingen, San Diego, CA). HEL-transgenic B cells were detected by staining with HEL-biotin followed by streptavidin Cy-Chrome (PharMingen). HEL-biotin was prepared using biotinamidocaproate N-hydroxysuccinimide (Sigma, St. Louis, MO) and standard protocols.

Analysis of Intracellular Calcium Ion $Ca^{2+}i$ Fluxes

Analysis was performed as described (Greimers et al., 1996). Splenocytes (10^7 cells/ml) were loaded with fluo-3/AM (Molecular Probes, Eugene, OR), stained with anti-B220 MAb, washed twice, and diluted to 10^6 cells/ml with 10 mM 4-(2-hydroxyethyl)piperazin-1-ethanesulfonic acid (HEPES)-buffered salt containing 137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO4, 5 mM glucose, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 g/liter BSA (pH 7.4). Cell suspensions were incubated at 37.5°C for 15 min before flow cytometry. After baseline unstimulated measurements, a stimulus was added to the sample on the flow cytometer. A23187 (Sigma, St. Louis, MO), an antibiotic calcium ion ionophore, was used at 10 μM, HEL (Sigma, St. Louis, MO) was used at 100 ng/ml, and goat anti-mouse IgM, $F(ab')_2$ fragment, (Jackson ImmunoResearch Laboratories Inc.,ME) was used at 10 μg/ml. Total time of acquisition per sample was 300 s.

BrdU Labeling and Analysis

Experimental mice were injected intraperitoneally with 0.6 mg of BrdU (Sigma) in 0.2 ml sterile, pyrogen-free Hank's balanced salt solution at 12 hr intervals for 7 days. BrdU incorporation was determined according to published procedure (Allman et al., 1992). Briefly, lymph node or splenic MNCs were purified as described

above and then permeabilized by drop-wise addition of ice-cold 95% ethanol, washed, and fixed in phosphate-buffered saline with 1% paraformaldehyde plus 100 U/ml DNAse to partially degrade and denature their chromatin. Subsequently, treated cells were stained with FITC-labeled antibody to BrdU (MAb B44; Becton Dickinson) and counter-stained with HEL-biotin (as described above) and B220 MAb; cells were analyzed by two-color flow cytometry.

In Vitro Activation Assay

Splenic mononuclear cells were purified and cultured overnight with 100 ng/ml of hen egg lysozyme as described (Fischer et al., 1998a). To detect activated HEL-binding transgenic B cells, splenic MNCs were stained with HEL-biotin and B220-FITC followed by streptavidin-Cy-Chrome and counterstained with PE-labeled anti-CD86 (B7–2) MAbs (all obtained from PharMingen).

Anti-Nuclear Antibodies

Anti-nuclear antibodies (ANA) were determined using Hep2-coated slides (Binding Site Ltd, Birmingham, England) incubated with serial dilutions of mouse sera in PBS. After washing, goat anti-mouse IgG labeled with FITC was used as secondary antibody (Sigma). After mounting with 10% glycerol in PBS, slides were viewed by fluorescent microscopy and the last positive dilution determined by comparison with wild-type mouse control serum at 1:50 dilution and mean of titers were calculated for each group.

Detection of Anti-dsDNA Autoantibodies

Anti-dsDNA-ELISA was performed as described (Emlen et al., 1990). Briefly, avidin (Sigma; affinity purified from egg white) was diluted in ELISA coating buffer (0.05 M carbonate [pH 9.6]) to 10 μg/ml and incubated overnight at 4°C in 96-well microtiter plate. Wells were washed three times with PBS + 0.02% Tween (PBST) between each step. Next, plates were incubated 1 hr at room temperature (or overnight at 4°C) with photobiotinylated DNA (1 μg/ml) (prepared according to manufacturer's protocol; Sigma) diluted in PBS and then blocked with 1% BSA/PBS for 1 hr at 37°C. Serum samples were diluted in 1% BSA/PBS and incubated for 2 hr at 37°C. Goat anti-mouse whole molecule IgG-AP conjugate (Sigma) or isotype-specific AP-conjugated antibodies (Southern Biotechnology Associates, Inc.) were diluted 1:750 in 1% BSA/PBS and incubated for 1 hr at 37°C. AP substrate (Sigma) dissolved in substrate buffer was added and optical density at 450 nm was read 45 minutes later. To convert optical density to concentration of IgG, samples were compared to that of mouse IgG of known concentration in serial dilutions.

Staining of Kidneys

Kidneys were either fixed in buffered formalin and stained with hematoxylin and eosin or periodic acid-Schiff (PAS) or quick frozen in OCT for cryosections as described (Ahearn et al., 1996). Immunostaining was performed on cryosections using goat anti-mouse IgG FITC-labeled (Sigma) diluted in 1% BSA/PBS + 0.02% Tween (1:400). The slides were incubated for 60 minutes in a humid chamber at room temperature. After washing with PBS, slides were covered with mounting media and viewed under fluorescence microscope (Leica DM-LB). The deposition of immune complexes was determined at 400× magnification and the number of affected glomeruli were counted per 100 glomeruli; $n \geq 3$ mice per group. PAS and H and E stained kidney sections were assigned scores based on glomerular enlargement and hypercellularity using the following numbering: 0, <10% of glomeruli affected; 1, 10%–30% glomeruli affected; 2, 30%–75% glomeruli affected; 3, 75%–95% glomeruli affected; and 4, >95% glomeruli affected.

Statistical Analysis

Student's t test assuming unequal variance was used for comparisons. Results are reported as means ± standard deviation, except where noted SEM values are used.

Acknowledgments

We would like to thank Drs. Garnett Kelsoe and Abul Abbas for critical discussions and reading of the manuscript. We especially thank Dr. E. Tsitsikov (Children's Hospital, Boston) for help with the $Ca^{2+}i$ mobilization assays and Dr. Henry Warren (Center for Animal Resources and Comparative Medicine, Harvard Medical School) for examining the pathology of kidney sections. We also thank Dr. Minghe Ma, Ms. Junrong Xia, and Ms. Xiaoning Zhou for help with the mice. M. C. C. is supported by grants from the National Institutes of Health and Cambridge Antibodies, Inc., Cambridge, United Kingdom.

Received August 8, 1998; revised Octobter 8, 1998.

References

Allman, D.M., Ferguson, S.E., and Cancro, M.P. (1992). Peripheral B cell maturation II: heat-stable antigen high splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. J. Immunol. *151*, 431–444.

Agnello, V. (1978). Association of systemic lupus erythematosus and SLE-like syndromes with hereditary and acquired complement deficiency states. Arthritis Rheum. *21*, 146–152.

Ahearn, J.M., Fischer, M.B., Croix, D., Goerg, S., Ma, M., Xia, J., Zhou, X., Howard, R.G., Rothstein, T.L., and Carroll, M.C. (1996). Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. Immunity *4*, 251–262.

Botto, M., Dell'Agnola, C., Bygrave, A.E., Thompson, E.M., Cook, H.T., Petry, F., Loos, M., Pandolfi, P.P., and Walport, M.J. (1998). Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat. Genet. *19*, 56–59.

Carroll, M.C. (1998a). The role of complement and complement receptors in induction and regulation of immunity. Annu. Rev. Immunol. *16*, 545–568.

Carroll, M.C. (1998b). The lupus paradox. Nat. Genet. *19*, 3–4.

Casciola-Rosen, L.A., Anhalt, G., and Rosen, A. (1994). Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J. Exp. Med. *179*, 1317–1330.

Chan, O., and Shlomchik, M.J. (1998). A new role for B cells in systemic autoimmunity: B cells promote spontaneous T cell activation in MRL-lpr/lpr mice. J. Immunol. *160*, 51–59.

Goodnow, C.C., Crosbie, J., Jorgensen, H., Brink, R.A., and Basten, A. (1989). Induction of self-tolerance in mature peripheral B lymphocytes. Nature *342*, 385–390.

Cooke, M.P., Heath, A.W., Shokat, K.M., Zeng, Y., Finkelman, F.D., Linsley, P.S., Howard, M., and Goodnow, C.C. (1994). Immunoglobulin signal transduction guides the specificity of B cell-T cell interactions and is blocked in tolerant self-reactive B cells. J. Exp. Med. *179*, 425–438.

Cotran, R.S., Kumar, V., and Robbins, S.L. (1994). Pathologic Basis of Disease (Philadelphia: W.B. Saunders).

Coutinho, A., Kazatchkine, M.D., and Avrameas, S. (1995). Natural autoantibodies. Curr. Opin. Immunol. *7*, 812–818.

Croix, D.A., Ahearn, J.M., Rosengard, A., Han, S.-H., Ma, M., and Carroll, M.C. (1996). Antibody response to a T-dependent antigen requires B cell expression of complement receptors. J. Exp. Med. *183*, 1857–1864.

Cyster, J.G., and Goodnow, C.C. (1995). Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in B lymphocytes and determines thresholds for negative selection. Immunity *2*, 13–24.

Cyster, J.G., Healy, J.I., Kishihara, K., Mak, T.W., Thomas, M.L., and Goodnow, C.C. (1996). Regulation of B-lymphocyte negative and positive selection by tyrosine phosphatase CD45. Nature *381*, 325–328.

Emlen, W., Jarusiripat, P., and Burdick, G. (1990). A new ELISA for the detection of double-stranded DNA antibodies. J. Immunol. Methods *132*, 91–101.

Erikson, J., Radic, M.Z., Camper, S.A., Hardy, R.R., Carmack, C.,

and Weigert, M. (1991). Expression of anti-DNA immunoglobulin transgenes in non-immune mice. Nature *349*, 331–334.

Fearon, D.T., and Carter, R.H. (1995). The CD19/CR2/TAPA-1 complex of b lymphocytes: linking natural to acquired immunity. Annu. Rev. Immunol. *13*, 127–149.

Fischer, M., Ma, M., Goerg, S., Zhou, X., Xia, J., Finco, O., Han, S., Kelsoe, G., Rothstein, T.L., Howard, J., et al. (1996). Regulation of the B cell response to T-dependent antigens by classical pathway complement. J. Immunol. *157*, 549–556.

Fischer, M.B., Goerg, S., Shen, L.M., Prodeus, A.P., Goodnow, C.C., Kelsoe, G., and Carroll, M.C. (1998a). Survival of germinal center B cells is dependent on expression of CD21/CD35. Science *280*, 582–585.

Fischer, M.B., Ma, M., Hsu, N., and Carroll, M.C. (1998b). Local synthesis of C3 within the splenic lymphoid compartment can reconstitute the impaired immune response of C3-deficient mice. J. Immunol. *160*, 2619–2625.

Fulcher, D.A., and Basten, A. (1994). Reduced life span of anergic self-reactive B cells in a double-transgenic model. J. Exp. Med. *179*, 125–134.

Goodnow, C.C. (1996). Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. Proc. Natl. Acad. Sci. USA *93*, 2264–2271.

Goodnow, C., Cyster, J.G., Hartley, S.B., Bell, S.E., Cooke, M.P., Healy, J.I., Akkaraju, S., Rathmell, J.C., Pogue, S.L., and Shokat, K.P. (1995). Self-tolerance checkpoints in B lymphocyte development. Adv. Immunol. *59*, 279–369.

Greimers, R., Trebak, M., Moutschen, M., Jacobs, N., and Boniver, J. (1996). Improved four-color flow cytometry method using fluo-3 and triple immunofluorescent for analysis of intracellular calcium ion (Ca2+) fluxes among mouse lymph node B- and T-lymphocyte subsets. Cytometry *23*, 205–217.

Inaoki, M., Sato, S., Weintraub, B.C., Goodnow, C.C., and Tedder, T.F. (1997). CD19-regulated signaling thresholds control peripheral tolerance and autoantibody production in B lymphocytes. J. Exp. Med. *186*, 1923–1931.

Klaus, G.B., Humphrey, J.H., Kunkl, A., and Dongworth, D.W. (1980). The follicular dendritic cell: its role in antigen presentation in the generation of immunological memory. Immunol. Rev. *53*, 3–29.

Korb, L.C., and Ahearn, J.M. (1997). C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes. J. Immunol. *158*, 4525–4528.

Michet, C.J., Jr., McKenna, C.H., Elveback, L.R., Kaslow, R.A., and Jurland, L.T. (1985). Epidemiology of systemic lupus erythematosus and other connective tissue diseases in Rochester, Minnesota, 1950 through 1979. Mayo Clin. Proc. *60*, 105–113.

Molina, H., Holers, V.M., Li, B., Fung, Y., Mariathasan, S., Goellner, J., Strauss-Schoenberger, J., Karr, R.W., and Chaplin, D.D. (1996). Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2. Proc. Natl. Acad. Sci. USA *93*, 3357–3361.

Nemazee, D.A., and Burki, K. (1989). Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. Nature *337*, 562–566.

Qin, D., Wu, J., Carroll, M.C., Burton, G.F., Szakal, A.K., and Tew, J.G. (1998). Antibody production and B cell-FDC communication via CD21-CD21 ligand. J. Immunol. *161*, 4549–4554.

Pepys, M.B. (1972). Role of complement induction of the allergic response. Nature *237*, 157–159.

Rathmell, J.C., Cooke, M.P., Ho, W.Y., Grein, J., Townsend, S.E., Davis, M.M., and Goodnow, C.C. (1995). CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. Nature *376*, 181–184.

Takahashi, T., Tanaka, M., Brannan, C.I., Jenkins, N.A., Copeland, N.G., Suda, T., and Nagata, S. (1994). Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell *76*, 969–976.

Theofilopoulos, A.N., and Dixon, F.J. (1985). Murine models of systemic lupus erythematosus. Adv. Immunol. *37*, 269–390.

Walport, M.J., and Morgan, B.P. (1991). Complement deficiency and disease. Immunol. Today *12*, 301–306.